Lab 4
Enzyme Immunoassays

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Enzyme Immunoassays

- Enzyme immunoassays (EIAs) can be used for detection of either antigens or antibodies in serum and other body fluids of the patient.
- In EIA techniques, antigen or antibody labeled with enzymes are used. Alkaline phosphatase, horseradish peroxidase, and galactosidase.
- The commonly used EIAs are enzyme-linked immunosorbent assays (**ELISA**).
- These assays involve the use of an immunosorbent specific to either the antigen or antibody. Following the antigen–antibody reaction, chromogenic substrate specific to the enzyme peroxidase, alkaline phosphatase, etc.) is added.
- The reaction is detected by reading the optical density. Changing color
- Usually, a standard curve based on known concentrations of antigen or antibody is prepared from which the unknown quantities are calculated.

There are different types of ELISA

(a) Indirect ELISA,

(b) Sandwich ELISA,

(c) Competitive ELISA.

Antibody detection

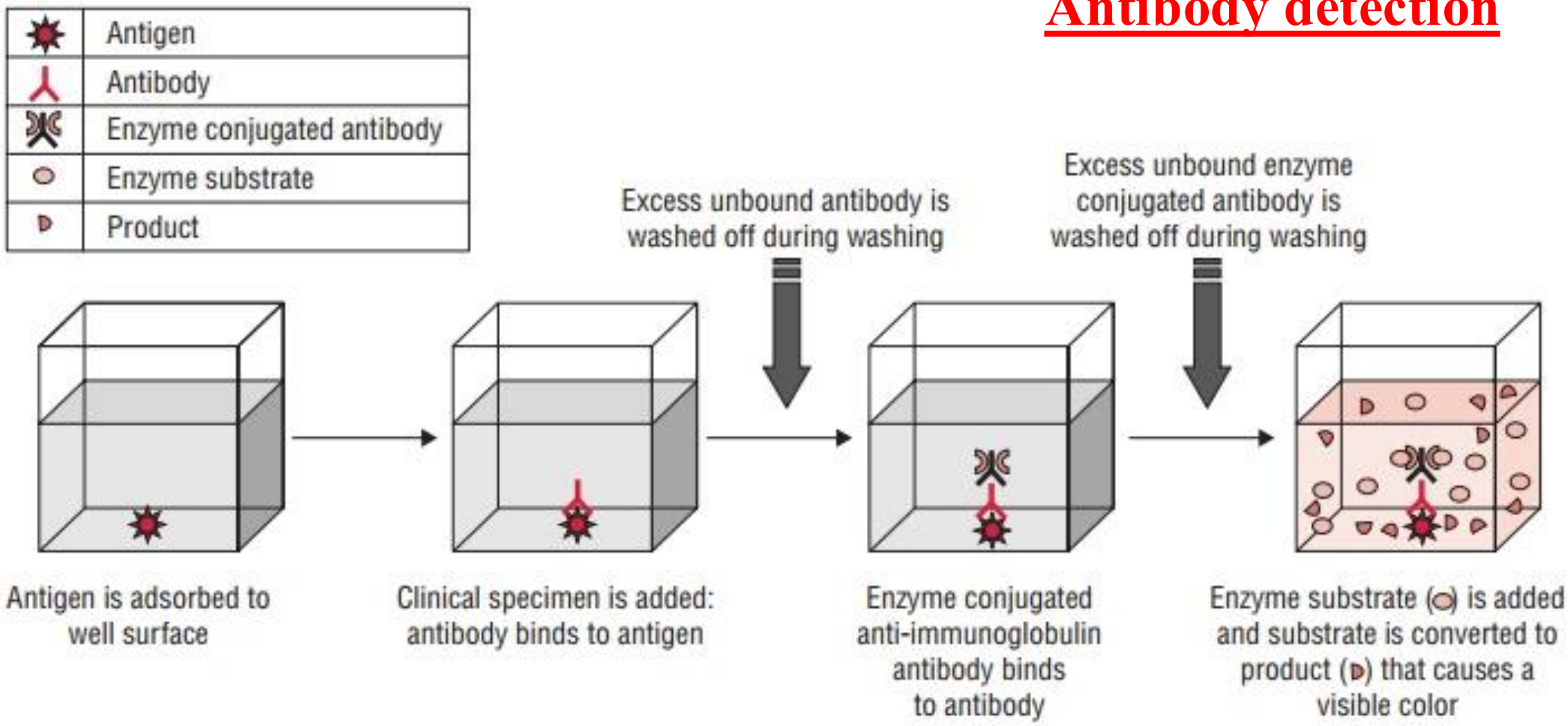


FIG. 14-14. Indirect ELISA test.

Antigen Detection

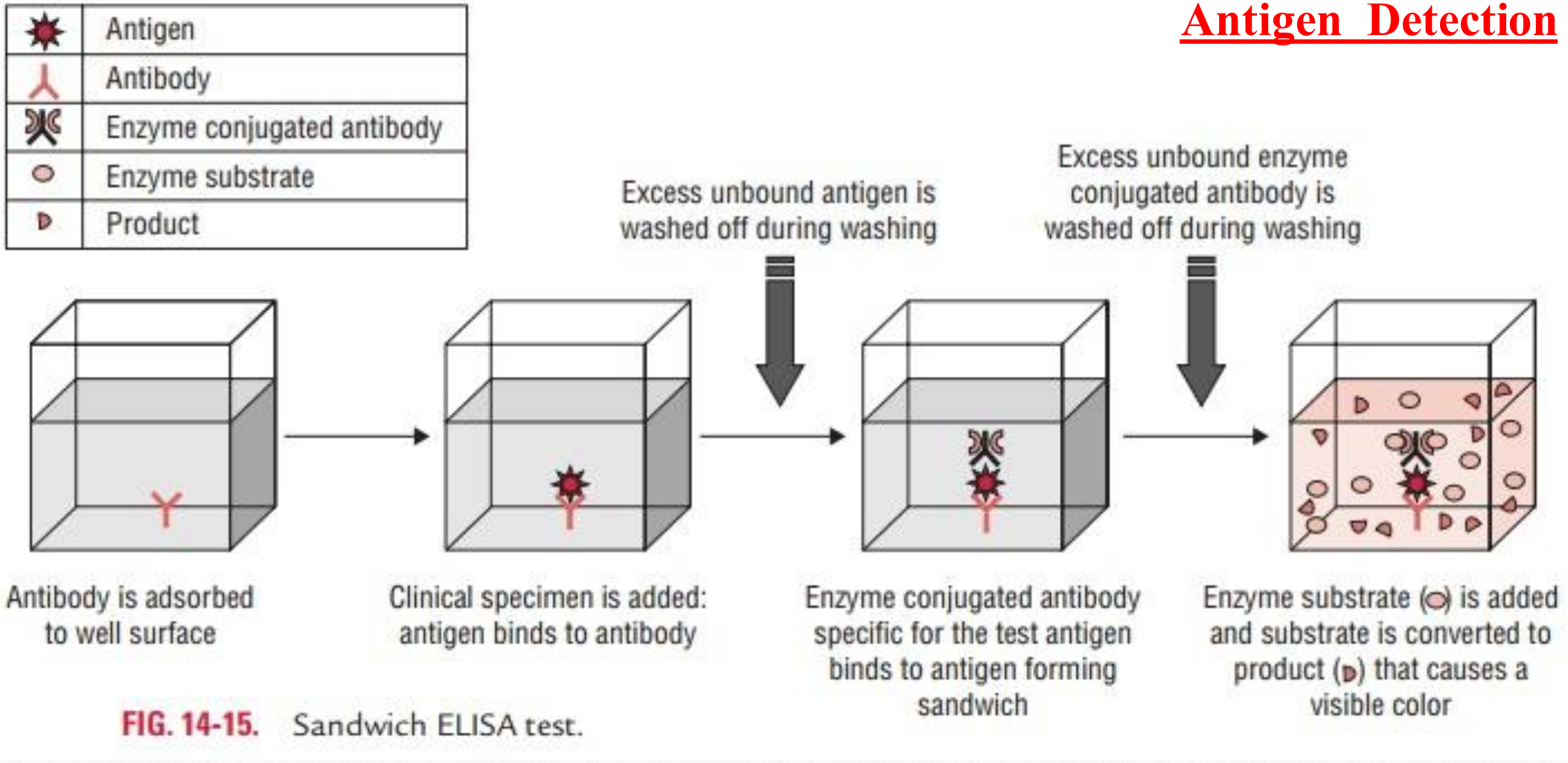
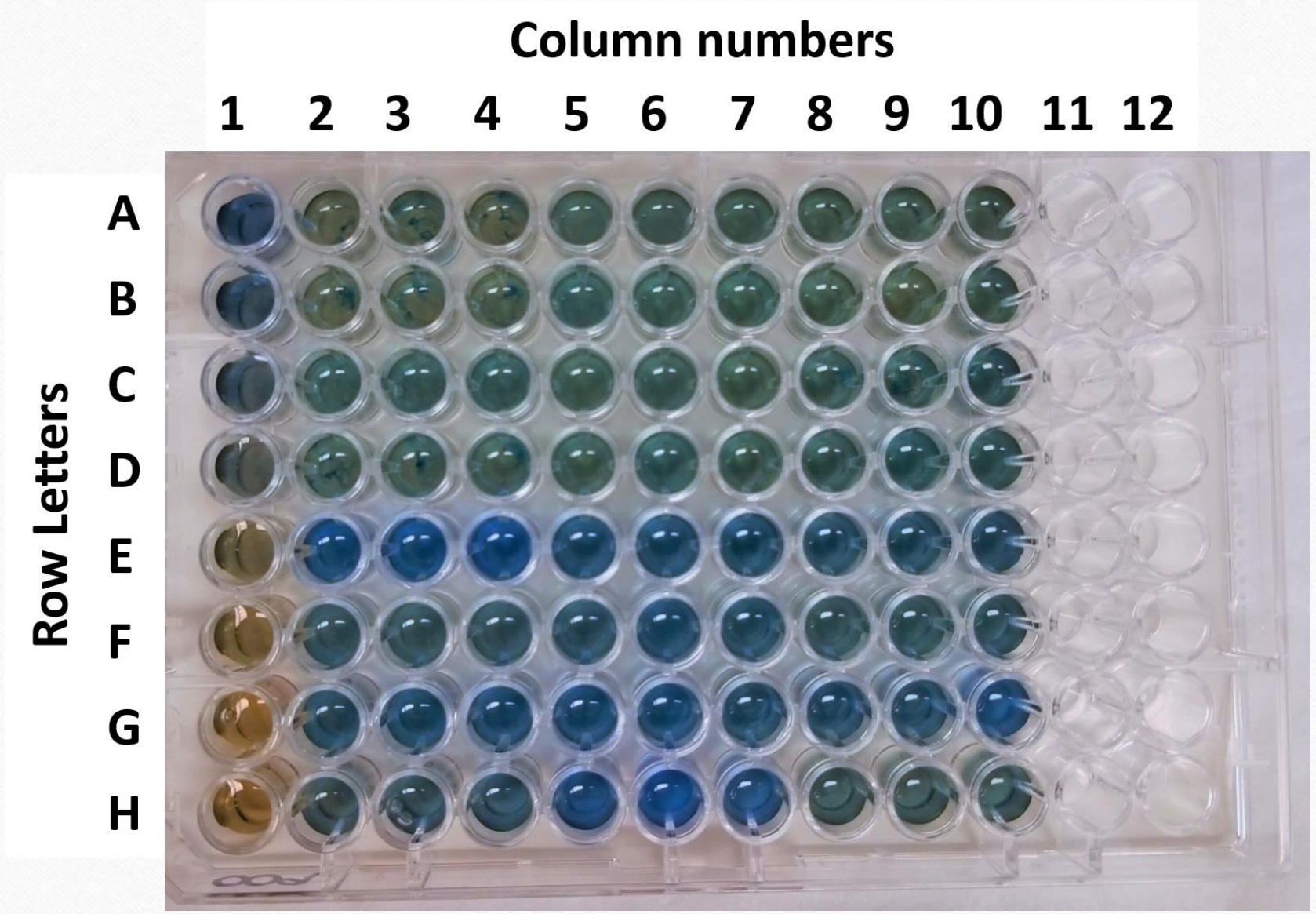


FIG. 14-15. Sandwich ELISA test.

ELISA plate

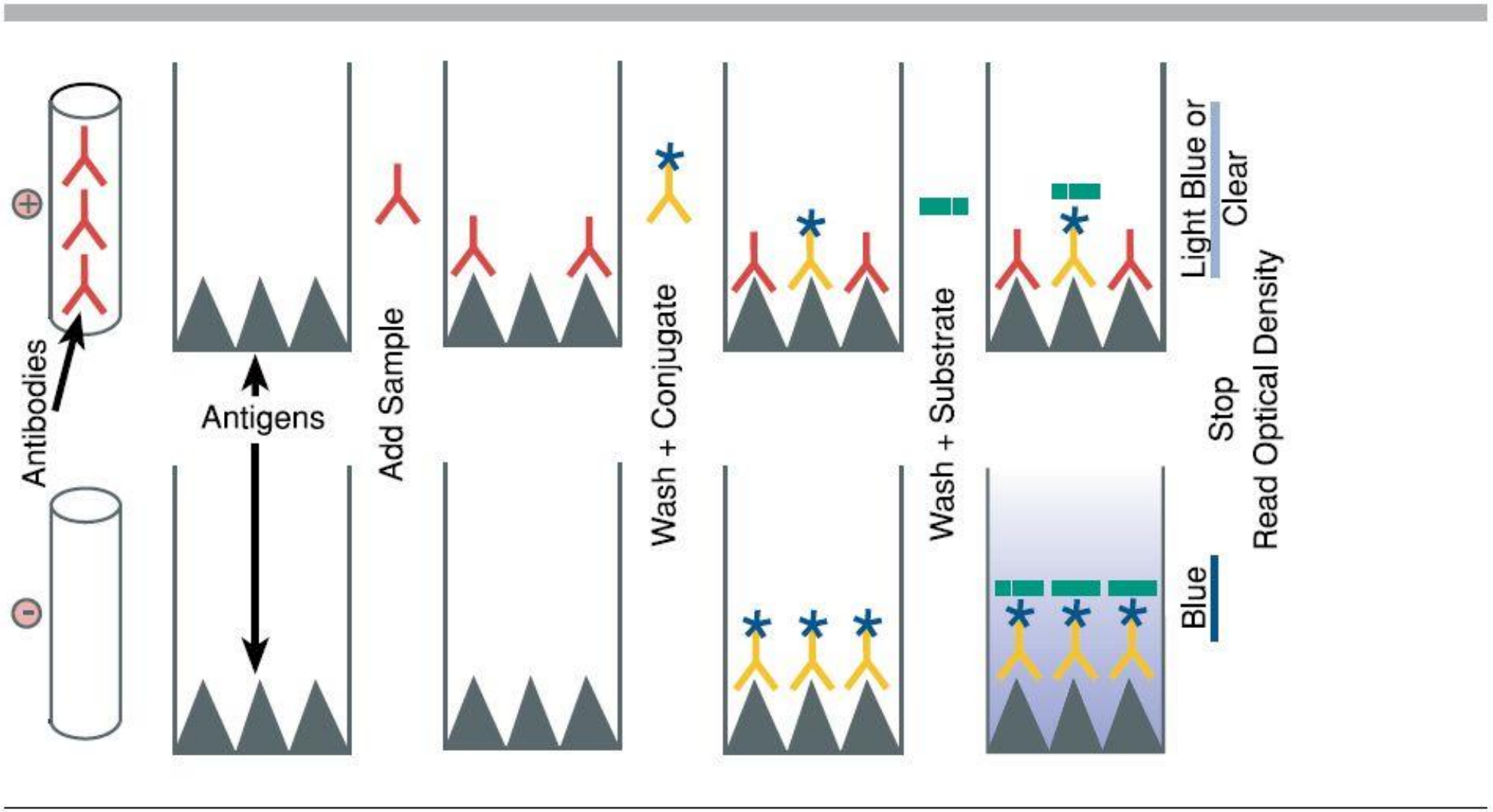


Competitive ELISA

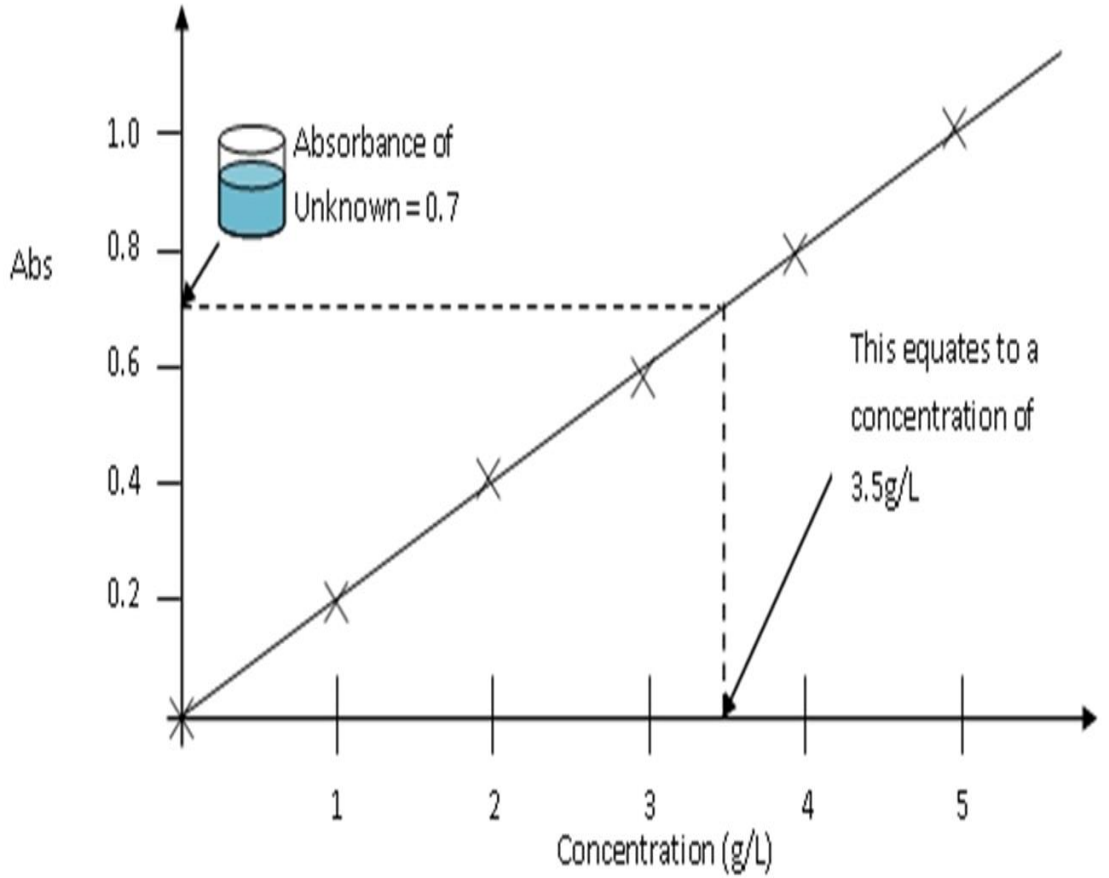
- Used for the estimation of **antibodies** present in a specimen, such as serum.
- Principle of the test is that two specific antibodies, one conjugated with enzyme and the other present in test serum (if serum is positive for antibodies), is used.
- Competition occurs between the two antibodies for the same antigen.
- Appearance of color indicates a negative test (absence of antibodies), while the absence of color indicates a positive test (presence of antibodies).

In this test, the microtiter wells are coated with HIV antigen.

- The sera to be tested are added to these wells and incubated at 37°C and then washed.
- If antibodies are present in the test serum, antigen–antibody reaction occurs.
- The antigen– antibody reaction is detected by adding enzyme-labeled-specific HIV antibodies.
- In a positive test, no antigen is left for these antibodies to act.
- Hence, the antibodies remain free and are washed away during the process of washing.
- When substrate is added, no enzyme is available to act on it.
- Therefore, positive result indicates no color reaction. In a negative test, in which no antibodies are present in the serum, antigen in the coated wells is available to combine with enzyme-conjugated antibodies and the enzyme acts on the substrate to produce color.



Competitive ELISA


Abs
1.0
0.8
0.6
0.4
0.2
Absorbance of
Unknown = 0.7
This equates to a
concentration of
3.5g/L
1
2
3
4
5
Concentration (g/L)

Western Blotting Techniques

- Blotting is a technique by which a macromolecule such as DNA, RNA, or protein is resolved in a gel matrix, transferred to a solid support, and detected with a specific probe.
- These powerful techniques allow us to identify and characterize specific molecules in a complex mixture of related molecules
- Some of the more common techniques include:

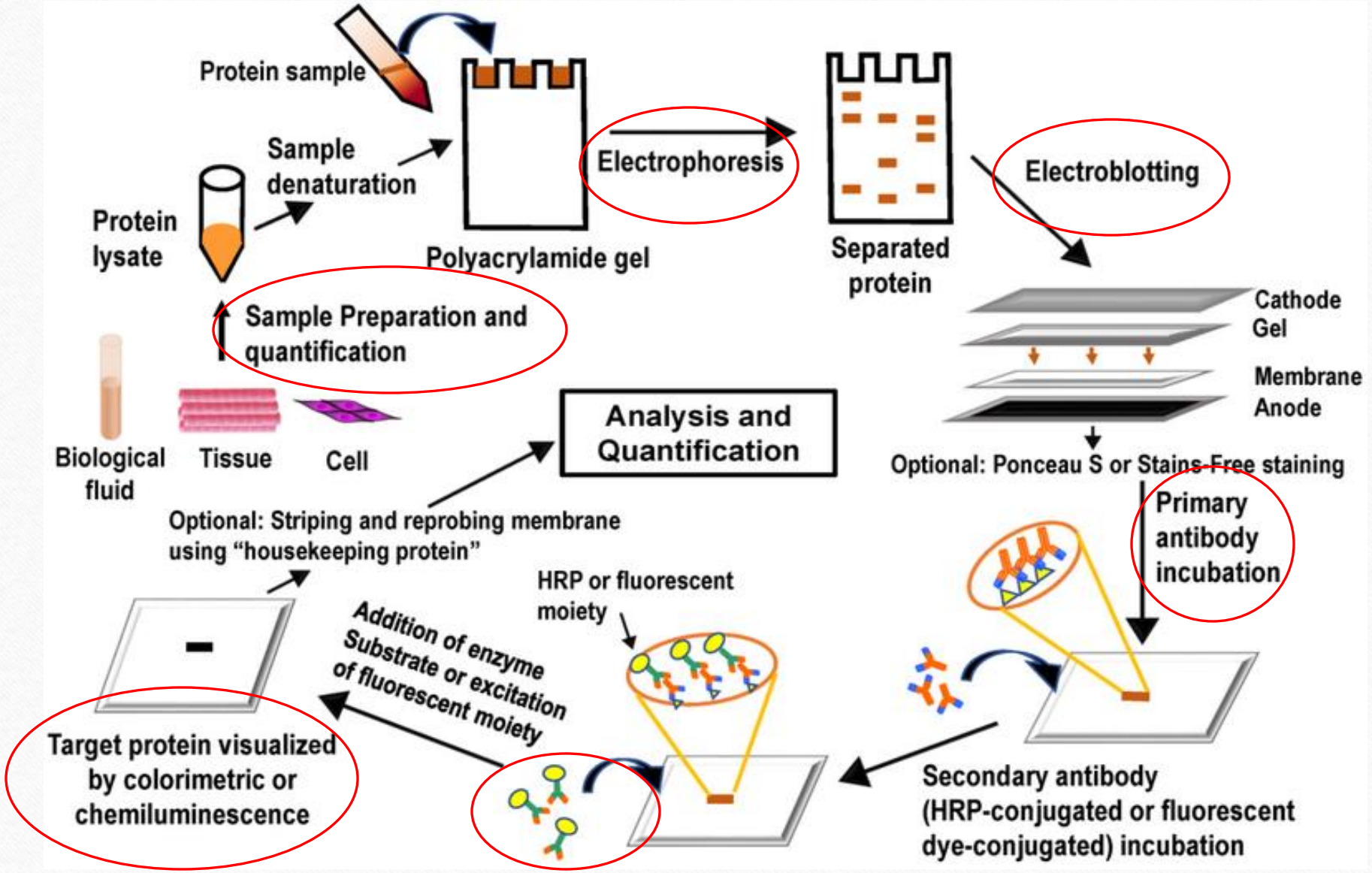
✓ Southern blotting (DNA)
✓ Northern blotting (RNA)
✓ Western blotting (for protein)

Western Blot Applications for Medical Diagnosis

- For HIV
  - confirmatory HIV-test to detect anti-HIV antibody in a human serum sample
- For HBV
  - confirmatory test for Hepatitis B infection
- For HSV
  - detection of Herps infections

Steps involved in western blotting

1. Sample preparation
2. Gel Electrophoresis sing SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) to separate a complex protein mixture by size
3. Blotting (or transfer) to membrane
4. Blocking; a crucial step using protein solutions (like milk or BSA) to saturate empty spots on the membrane after protein transfer, preventing antibodies from sticking non-specifically and causing high background noise
5. Antibody Probing, the core detection step where a membrane with transferred proteins is incubated with a primary antibody (specific to your target protein) and then a labeled secondary antibody (binding the primary) to reveal protein presence, size, and quantity,
6. Detection, finally using a substrate or light/fluorescence detection to visualize protein bands, revealing protein presence and quantity


Protein sample
Sample denaturation
Protein lysate
Polyacrylamide gel
Electrophoresis
Separated protein
Electroblotting
Cathode
Gel
Membrane
Anode
Optional: Ponceau S or Stains-Free staining
Primary antibody incubation
Secondary antibody (HRP-conjugated or fluorescent dye-conjugated) incubation
HRP or fluorescent moiety
Addition of enzyme Substrate or excitation of fluorescent moiety
Target protein visualized by colorimetric or chemiluminescence
Optional: Striping and reprobing membrane using "housekeeping protein"
Analysis and Quantification
Sample Preparation and quantification
Biological fluid
Tissue
Cell

Immunofluorescence

The property of certain dyes absorbing light rays at one particular wavelength (ultraviolet light) and emitting them at a different wavelength (visible light) is known as fluorescence. Fluorescent dyes, such as fluorescein **isothiocyanate** and **lissamine rhodamine**, can be tagged with antibody molecules. They emit blue-green and orange-red fluorescence, respectively under ultraviolet (UV) rays in the fluorescence microscope. This forms the basis of the immunological test. Immunofluorescence tests have wide applications in research and diagnostics. These tests are broadly of two types:

1. Direct immunofluorescence test

2. Indirect immunofluorescence test

Direct immunofluorescence test
Is widely used for detection of bacteria, parasites, viruses, fungi, or other antigens in CSF, blood, stool, urine, tissues, and other specimens.

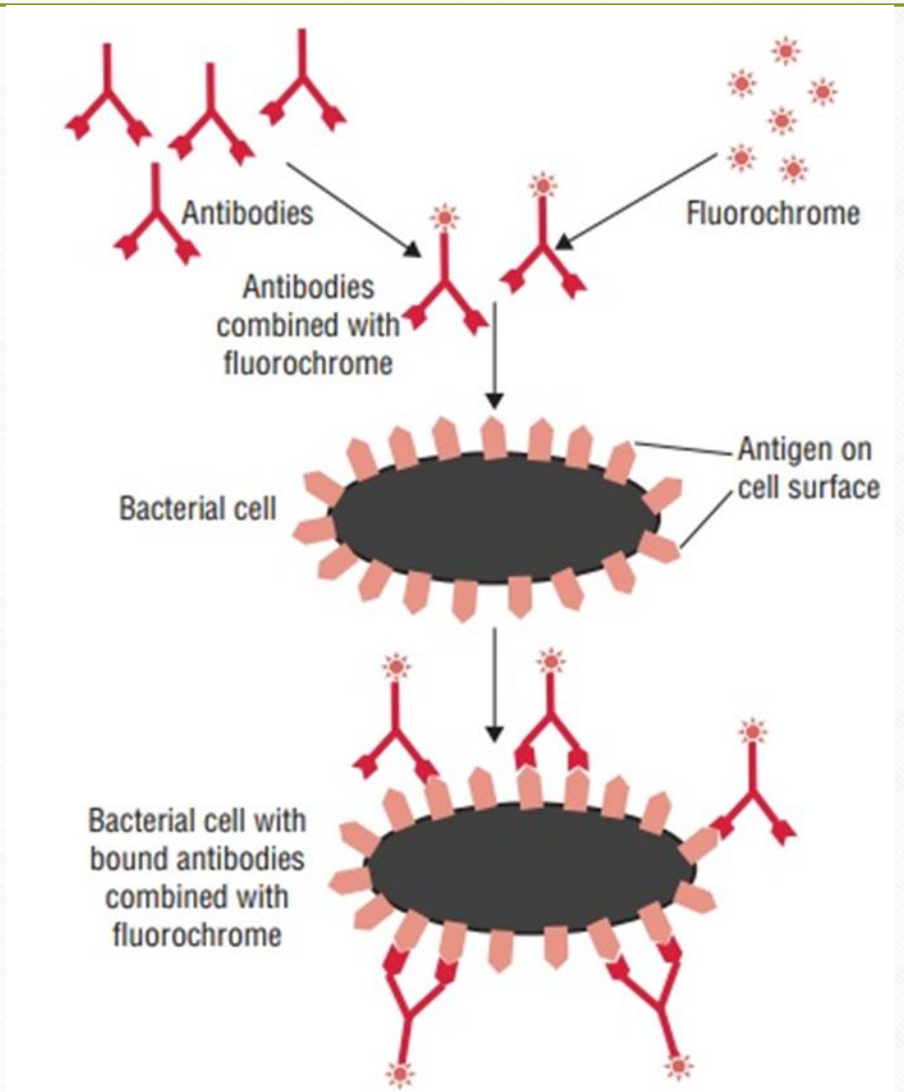
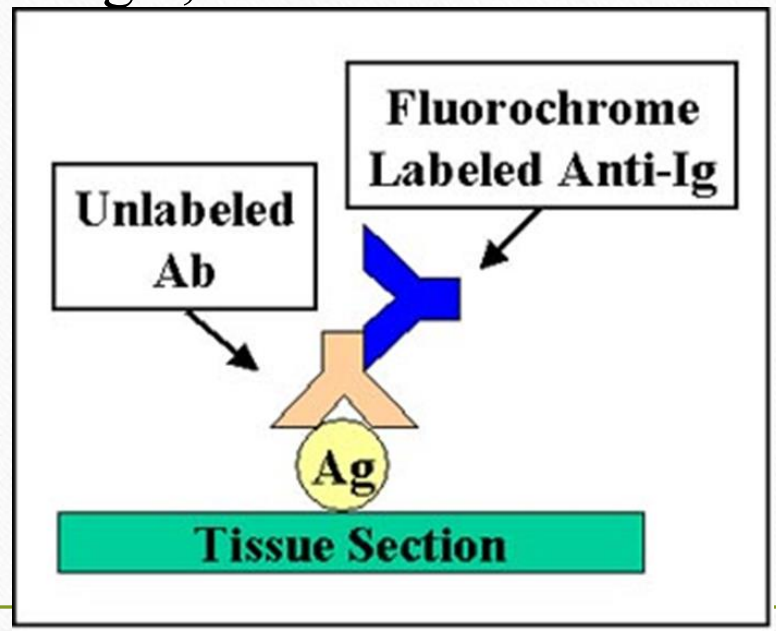


FIG. 14-13. Direct fluorescent antibody test.

Indirect immunofluorescence test

Indirect immunofluorescence is a two-stage process.

- **First stage**, a known antigen is fixed on a slide. Then the patient's serum to be tested is applied to the slide, followed by careful washing. If the patient's serum contains antibody against the antigen, it will combine with antigen on the slide.
- **Second stage**, the combination of antibody with antigen can be detected by addition of a fluorescent dye-labeled antibody to human IgG, which is examined by a fluorescence microscope.

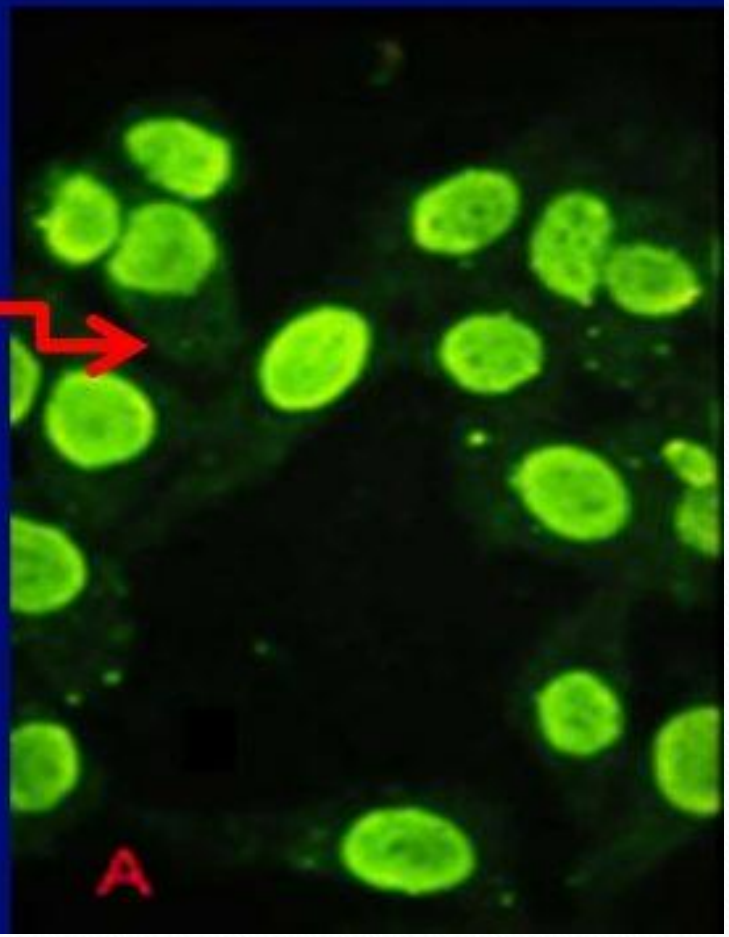


Test ANA in autoimmune diseases

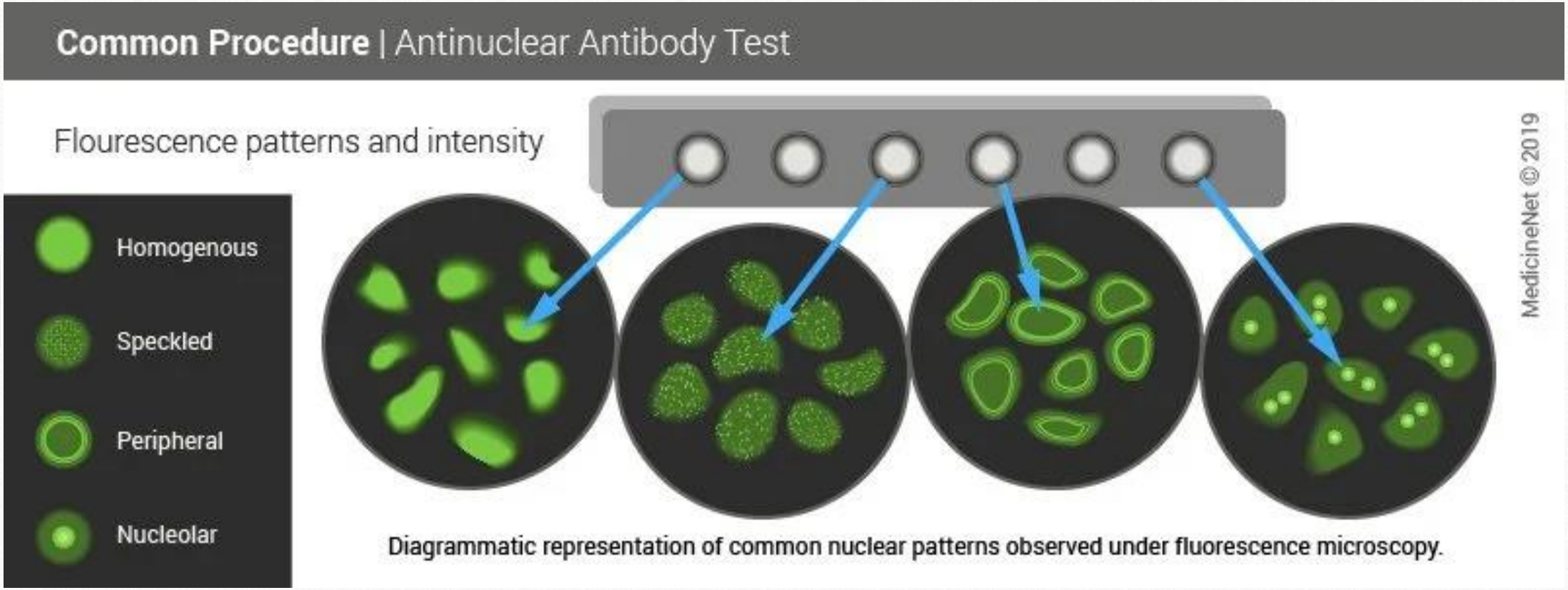
- Antinuclear antibodies (ANA) are a category of antibodies that targets the **nucleus** of other cells.
- The ANA blood test used to help diagnose certain autoimmune conditions, particularly **lupus**.
- Patient serum is added to a slide containing cells. If the patient has autoantibodies to the nuclei of the cells, they bind to the slide.
- After washing away any antibodies that don't bind, an antibody against human antibody is added.
- This antibody has radiolabeled molecules attached to it which, when viewed under Immunofluorescent microscope (light-up green).

Auto antibodies

- The anti-nuclear antibody (ANA) test is the best screening test for SLE and is determined by **immunofluorescence** or **ELISA** tests
- The ANA is positive in significant titer (usually 1:160 or higher) in virtually all patients with SLE



Patterns of ANA Test



Example

HOMOGENEOUS PATTERN

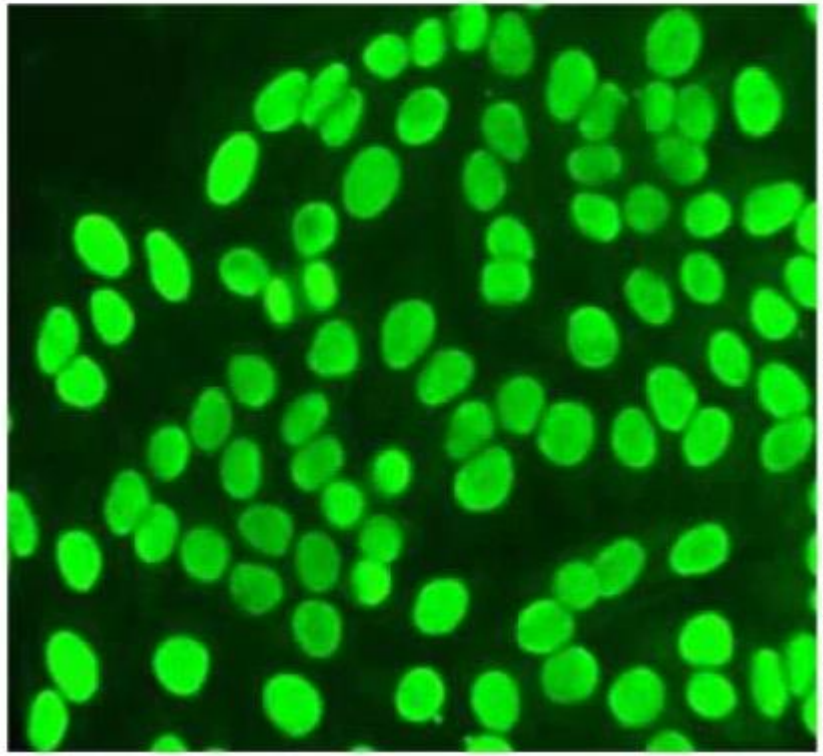
Uniform diffuse fluorescence staining of the entire nucleus in interphase cells.

ANTIGENS:

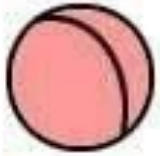
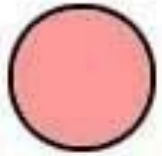
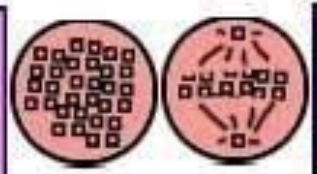
- DNA, DNA-histone

DISEASES:

- SLE (very specific)
- Drug-induced lupus
- RA **(Rheumatoid Arthritis)**
- Juvenile chronic arthritis
- Systemic sclerosis



ANA Patterns

Peripheral (rim)		Anti-DNA (not seen on HEP-2)	SLE
Homogeneous (diffuse)		Anti-DNA Anti-histone Anti-DNP (nucleosomes)	RA & SLE Misc. Disorders (anti-ssDNA)
Speckled		Anti-Sm & RNP Anti-Ro & La Anti-jo-1 & Mi-2) Anti-Sci-70	SLE & SS PM/DM PSS (Systemic)
Centromere		Anti-centromere	PSS (CREST)
Nucleolar		Anti-nucleolar	SLE & PSS

- Although sensitive, the fluorescence microscope is not an ideal tool to identify the detailed structure of the cell or tissue because of a low structural details.
- This problem has been overcome by new technologies including **confocal microscopy**.

- Antibody can be coupled to an electron-dense probe such as colloidal gold, and the location of antibody can be determined subcellular by means of an electron microscope.